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## **Nitrogen gas produces less behavioural and neurophysiological excitation than carbon dioxide in mice undergoing euthanasia**

Detotto, Carlotta ; Isler, Sarah ; Wehrle, Martin ; Vyssotski, Alexei L ; Bettschart-Wolfensberger, Regula ; Gent, Thomas C

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RESEARCH ARTICLE

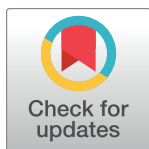
# Nitrogen gas produces less behavioural and neurophysiological excitation than carbon dioxide in mice undergoing euthanasia

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## Abstract

Carbon dioxide (CO<sub>2</sub>) is one of the most commonly used gas euthanasia agents in mice, despite reports of aversion and nociception. Inert gases such as nitrogen (N<sub>2</sub>) may be a viable alternative to carbon dioxide. Here we compared behavioural and electrophysiological reactions to CO<sub>2</sub> or N<sub>2</sub> at either slow fill or rapid fill in C57Bl/6 mice undergoing gas euthanasia. We found that mice euthanised with CO<sub>2</sub> increased locomotor activity compared to baseline, whereas mice exposed to N<sub>2</sub> decreased locomotion. Furthermore, mice exposed to CO<sub>2</sub> showed significantly more vertical jumps and freezing episodes than mice exposed to N<sub>2</sub>. We further found that CO<sub>2</sub> exposure resulted in increased theta:delta of the EEG, a measure of excitation, whereas the N<sub>2</sub> decreased theta:delta. Differences in responses were not oxygen-concentration dependent. Taken together, these results demonstrate that CO<sub>2</sub> increases both behavioural and electrophysiological excitation as well as producing a fear response, whereas N<sub>2</sub> reduces behavioural activity and central neurological depression and may be less aversive although still produces a fear response. Further studies are required to evaluate N<sub>2</sub> as a suitable euthanasia agent for mice.

## Introduction

Carbon dioxide (CO<sub>2</sub>) is the most commonly used gas euthanasia agent in mice due to rapid action, low cost and easy availability [1, 2]. However, multiple studies have demonstrated that carbon dioxide exposure may cause pain in humans as well as fear responses and aversion in rodents [3–6] therefore its use for euthanasia is controversial [7–12]. Alternative agents are sought to improve the welfare conditions of mice [12]. Inert gases induce unconsciousness and death by hypoxia due to the displacement of oxygen and have been investigated for humane euthanasia of rodents [13–15]. The formation of carbonic acid in the mucous membranes following CO<sub>2</sub> absorption is considered to cause pain and discomfort as reported in humans [3, 16], however inert gas absorption does not result in carbonic acid formation. Furthermore, increasing acidity in the blood from CO<sub>2</sub> is detected in the amygdala of mice and

**Competing interests:** The authors have declared that no competing interests exist.

results in a fear response [6]. Humans undergoing short term hypoxia in hypobaric conditions do not report pain or distress and have poor perception of changes in environmental conditions [17]. Whether the same effects occur during normobaric hypoxia is uncertain. Argon (Ar) is considered to be aversive to rats [18, 19] and mice [10] presumably because loss of consciousness occurs due to hypoxia alone, and has also been documented to cause dyspnoea or “air gasping” at hypoxic conditions, which is likely to be aversive [12]. Its use is considered unsatisfactory for euthanasia, as it is slow to induce death and it causes hyperreflexia in rats [14]. Nitrogen is an inert gas which is colourless, odourless and non-irritant, but it has received little attention as a potential euthanasia agent compared to other inert gases. The proposed mechanism of N<sub>2</sub>-induced loss of consciousness and death is also by hypoxia [20]. One study suggested that N<sub>2</sub> may be less aversive than Ar since it does not increase heart rate and mean arterial blood pressure [14]. Additionally, N<sub>2</sub> gas is relatively cheap and abundant in the environment and therefore safe to the operator and non-polluting, in contrast to CO<sub>2</sub>, which has a significant environmental impact [21]. However, there remains insufficient data on the behavioural effects of N<sub>2</sub> on mice to determine its usefulness as a euthanasia agent [20, 22].

The American Veterinary Medical Association (AVMA) euthanasia guidelines suggest an optimal fill rate between 10% and 30% chamber volume/min of CO<sub>2</sub> [23]. However, these guidelines are based on extrapolation from studies performed either in other paradigms, such as comparison between pre-filled chamber and gradual induction [24], preference and approach-avoidance testing [7, 25, 26], or in other species such as rats [27], cats [28] and humans [29], without any direct pain or distress measurement specifically in mice. Recent investigations in mice have drawn different conclusions as to whether rapid (80% chamber volume/min) or slow (20–30% chamber volume/min) fill rates of CO<sub>2</sub> are optimal for aversion in mice [8, 30, 31], whereas there are no reports of optimal fill rates for N<sub>2</sub>.

Here, we sought to determine firstly, whether N<sub>2</sub> is a less aversive alternative to CO<sub>2</sub> for gas euthanasia of mice, and secondly, whether slow fill is preferable to rapid fill using video tracking technology to assess behaviour (locomotion, jumping and freezing) in grouped animals. We further examined the brain activity during gas exposure in single animals chronically instrumented with EEG electrodes to test neurological excitation.

## Methods

### Animals

We used adult male (n = 135) and female (n = 119) C57Bl/6j mice (Charles Rivers Laboratories, Germany) housed in cages (Makrolon Type II long cage 365 x 205 x 140mm; Tecniplast) as litter-mates from 4 to 8 mice per cage as determined by national legislation (Tierschutzverordnung, Art. 10, Anh. 3). Cages were provided with a standard softwood bedding (Safe Lab), a piece of tissue paper (Lucart) and a resting box (Tecniplast). The median age of mice at experiment was 7.3 (6–15) weeks old and weight was 23.5 (20–25) grams. Animals were kept on a standard 12:12 light:dark cycle (lights on 08:00) and given standard rodent food (Granovit 3436) and autoclaved bottled water *ad libitum*. All experiments were performed during the light period. Animals were housed by sex from weaning at the age of three weeks and had minimal handling solely for husbandry purposes prior to experimentation. Instrumented animals were housed individually from the time of instrumentation until experimentation, in adjacent transparent cages with holes to allow diffusion of sound and smell. This was done to reduce stress associated with single housing.

### Instrumentation

To investigate brain activity associated with gas exposure, a separate cohort of animals were chronically instrumented for recording EEG and EMG (n = 6 per group). Animals were

anaesthetised in isoflurane (Isoflo, Abbott, 3% volume) in oxygen (flow rate 1litre/minute) and positioned in a stereotaxic frame, as previously reported [15]. Buprenorphine (100 µg/kg; Temgesic, Schering Plough), meloxicam (5 mg/kg; Metacam, Boehringer-Ingelheim) and 0.9% saline (10 ml/kg; B. Braun) were then administered subcutaneously. The hair was then shaved from the scalp and the skin aseptically prepared, first with iodine solution (Betadine) and 4 minutes later with 70% ethanol (B. Braun). Holes were drilled in the skull and three small jewellery screws (00 x 1/8", J.I. Morris, Southbridge, USA), soldered to 0.5mm stainless steel, PTFE wire (W3 Wire), were inserted above the dura (not penetrating brain tissue) as EEG electrodes. With respect to the cranial bregma suture, the ground electrode was placed + 4.0 mm anterior and + 1.0 mm lateral and the two recording electrodes—2.0 mm posterior and ± 2.0 mm lateral. The recorded signal was a differential voltage between the two posteriorly placed electrodes. The bare ends of two 0.5mm stainless steel, PTFE wires (W3 Wire) were implanted in the rhomboideus muscles of the neck as EMG electrodes. All electrodes were then soldered to a pin connector (M52-040023V2545, Harwin) and the implant sealed using methyl-methacrylate cement (Paladur). Animals were placed in a clean cage with fresh bedding, food and water and allowed to recover under a warming lamp (Philips). The cage was returned to the housing room when the animals were able to walk.

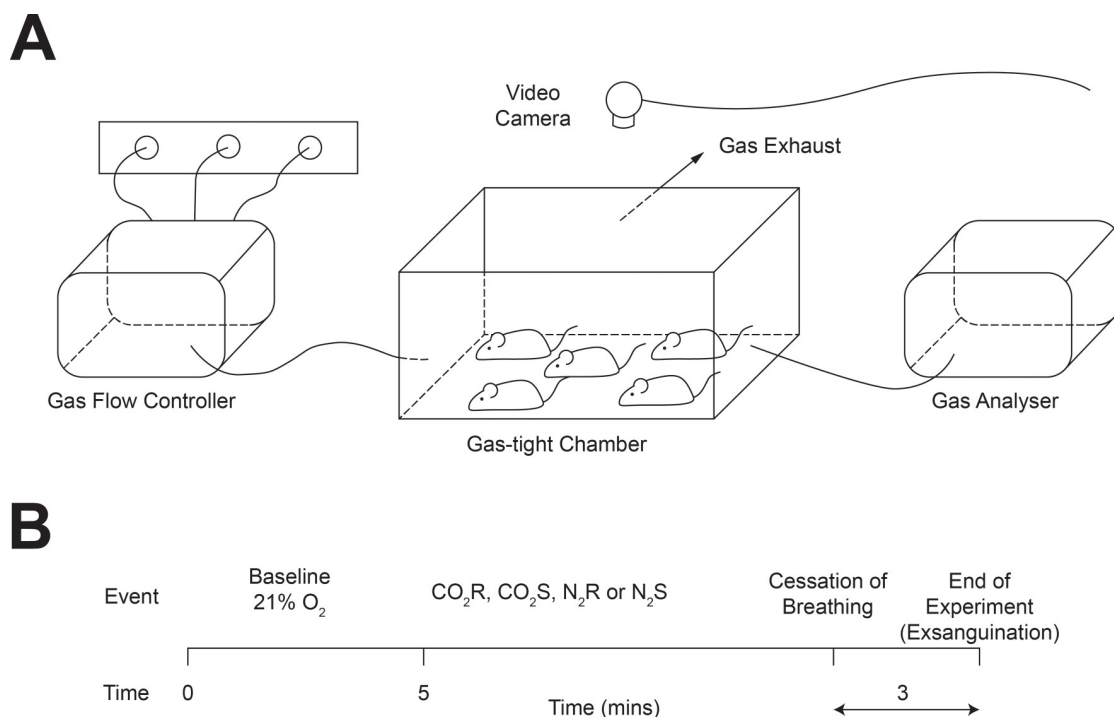
Animals were allowed two days to recover and were then habituated to wearing a miniature EEG recording device (Neurologger 2A; manufactured by one of the authors [32]) for 15 minutes each day for seven days whilst in the home cage. Experimentation was performed on the 9<sup>th</sup> day after surgery, during the light period (08:00–20:00). Data collected from different time-points in these experiments on some of the instrumented animals used in this study were reported in a previous study [15]. All of the animals in this investigation underwent only one gas exposure to either CO<sub>2</sub> or N<sub>2</sub> and one exposure to the chamber during their lifetime.

## Experiments/Recording

Experiments were performed in two separate formats. Firstly, uninstrumented animals underwent experimentation with their cage mates to assess the behaviour of animals in groups in response to CO<sub>2</sub> or N<sub>2</sub> exposure. Secondly, instrumented animals individually underwent experimentation to investigate the relationship between brain activity (measured by EEG) and behaviour in response to CO<sub>2</sub> or N<sub>2</sub> exposure.

Animals were randomly assigned to receive one of four gas exposures: CO<sub>2</sub> rapid (80% chamber volume per minute; CO<sub>2</sub>R), CO<sub>2</sub> slow (30% volume per minute; CO<sub>2</sub>S), N<sub>2</sub> rapid (N<sub>2</sub>R) or N<sub>2</sub> slow (N<sub>2</sub>S). Randomisation was performed by an automated randomisation function in Microsoft Excel (RAND() function). Briefly, the cage numbers of the animals were written in column A and a series of randomised numbers generated in column B. Column B was then sorted into descending order using the ZAR function, thus randomising the corresponding column A.

To prevent additional stress prior to experimentation, animals were allowed to become accustomed to the noise of the gas analyser and the experimental room whilst in the home cage for 30 minutes before starting the experiment. These measures were taken since transportation of animals the home cage itself may cause a physiological stress responses [33]. A single person was inside the experimental room to perform the experiments. Only instrumented animals were fitted with the EEG recording device on a table next to the home cage and returned to their home cage at least 30 minutes before the start of experimentation. Animals were handled solely for the purposes of fitting the EEG recording device (when appropriate) and also transfer to the chamber from the home cage which was placed immediately adjacent to it. All animals were individually lifted by their tail for transfer.



**Fig 1. Experimental design.** (A) Graphic representation of the experimental apparatus. The gas flow controller was calibrated to deliver a precise amount of each gas used and to switch from 21% oxygen to the treatment gas at the end of the baseline period. (B) Timeline of the experimental procedure. Mice were randomly assigned to one of four treatment groups: CO<sub>2</sub> or N<sub>2</sub> and either rapid (R—80% volume fill minute<sup>-1</sup>) or slow fill (S—30% volume fill minute<sup>-1</sup>). The experiment started with a 5-minute baseline recording at 21% O<sub>2</sub>. Either CO<sub>2</sub> or N<sub>2</sub> was then infused into the chamber as a 100% concentration of total gas inflow until cessation of breathing. The experiment was terminated three minutes after cessation of breathing and animals exsanguinated.

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All experiments were performed in a non-environmentally enriched acrylic gas-tight chamber (manufactured by the authors) measuring 400 x 400 x 150 mm for uninstrumented grouped animals (Fig 1A) or 250 x 250 x 150 mm for single instrumented animals. The gas inlet and port for measuring gas concentrations were placed 25 mm from the floor of the chamber and the gas exhaust 120 mm from the roof of the chamber. Animals were naïve to the experimental chamber at the start of experimentation. Instrumented animals underwent exposure as single animals, non-instrumented animals underwent exposure in groups of littermates from one cage (4–8 animals per group).

The start of experiments consisted of a 5-minute baseline in normal air (21% oxygen, 79% nitrogen). After 5 minutes, gas exposure began with both CO<sub>2</sub> and N<sub>2</sub> having a concentration of 100% of total gas inflow (Fig 1B). Gas flow rates for baseline were the same as for the exposure period (i.e.: 30% or 80% chamber volume per minute) and were accurately administered by a calibrated gas mixer (GSM-3, CWE Inc, USA). Oxygen concentrations were continuously sampled, 25 mm from the floor of the chamber, during experimentation at 1L per minute and recorded electronically (Rapidox 3100EA, Cambridge Sensotec) with a sampling rate of 1Hz. Electrophysiological data was acquired at 200 Hz. The experiment was terminated 3 minutes after cessation of breathing. The chamber was cleaned with 70% ethanol (B. Braun) and allowed to air dry before the next experiment.

### Behavioural analysis

During experiments, locomotion was recorded using a video camera and analysed post hoc with video tracking software (icy Mice Profiler Tracker [34]). In the software, the number of

pixels per centimetre were calculated using the known lengths of the sides of the chamber. The software then automatically detected the centre of each mouse, based on contrast to the background. The distance moved by each animal was then calculated for each second and the speed then calculated in centimetres per second. Normalised speeds were then calculated by dividing the speed during each second of the gas exposure by the average speed of the last 30 seconds of the baseline. In addition, we measured the number of vertical jumps, defined as vertical rapid jumps [35], both during baseline and gas exposure. Furthermore, we assessed freezing episodes, defined as a minimum period of two seconds of complete inactivity except that which is necessary for respiration, whilst the animal is standing with the head raised [6, 9], by visual scoring of videos throughout gas exposure. To ensure that all EEG and behavioural measurements were taken from the time when the animals were conscious, we determined the time point of loss of motion (LOM) which has been shown to occur momentarily before loss of righting reflex (LORR) [36, 37]—which is considered as a proxy for loss of consciousness in rodents [38]. This was determined from the calculated speed given by the video tracking software and visual scoring to assess that no purposeful movements were being made, such as walking, attempting to stand and grooming. LOM was considered to have occurred from the time point where locomotion speed was 0 cm/s and remained at this value for the remainder of the experiment. The number of vertical jumps, freezing episodes and LOM were scored by an observer who was blinded to the treatment of the animals. Experiments were terminated 3 minutes after the animals stopped breathing and animals were exsanguinated by cardiac puncture to ensure death. For instrumented animals, the EEG recording device then was detached from the animal and the data downloaded onto a computer.

## EEG analysis

Analysis of EEG recordings was performed in waveform analysis software (Spike 2, Cambridge Electronic Design, CED). Isoelectric EEG was defined as the point where no discernible oscillations were detectable, and was used as a marker of cessation of neocortical activity and therefore brain death [15]. EEG oscillations during slow-wave sleep and light general anaesthesia are characterised by slower rhythmic waves in the delta range (1–4.5 Hz) [38]. Whereas, wakefulness is characterised by faster theta rhythms (5–9 Hz) in the EEG. During exploratory behaviours and freezing behaviour, the frequency and power of theta oscillations further increases in the EEG of rodents [39, 40]. The power ratio between the two bands is considered to be an accurate measure of vigilance state and behavioural excitation [41, 42]. Band pass filters were applied to the raw EEG to create separate channels of theta (5–9 Hz) and delta (1–4 Hz) signals. The band passed signals were multiplied by themselves to create a signal of power ( $\mu V^2$ ) and theta:delta ratio calculated by dividing the theta power signal by the delta power signal, such that a value  $> 1$  indicates a predominant theta oscillation and a value  $< 1$  indicates a predominant delta oscillation. Theta:delta ratio was calculated in 1 second bins.

## Statistical analysis

Each animal was considered a single entity for data analysis whether housed in groups or alone. Data were analysed by one-way ANOVA with post-hoc Tukey's modification for parametric data and Kruskal-Wallis test for non-parametric data. P-values less than 0.05 were considered significant. Values for all mice within a cage were summated for comparison between baseline and treatment. Statistical analysis was performed using Graphpad Prism 6.0. Values in the text are reported as median and range for non-parametric and mean  $\pm$  s.e.m for parametric data. Locomotion data, jumps and freezing episodes were plotted as cumulative frequencies with respect to oxygen concentration. Locomotion data were normalised to activity



of the last 30 seconds of the baseline. Differences in distributions were calculated using the Kolmogorov-Smirnov test. Comparisons of baseline and treatment parameters were performed by paired T-test.

### Exclusion criteria

Animals were excluded from experimentation if they showed signs of pain assessed by a grimace scale score greater than 1 (as assessed from still photographs taken from cage-side video recordings [43]) or if body weight had not returned by 3 days after surgical implantation. Segments of electrophysiological data were excluded from analysis if they contained movement artefacts, defined as single deflections of more than 400  $\mu$ V lasting more than 200 ms during movement of the animal, or any segments with a saturated signal, where the recorded amplitude range was  $\pm 500 \mu$ V.

### Ethical approval

This work was approved by the Kanton Zürich Gesundheitsdirektion Veterinäramt. License numbers: 58/2014 and 051/2016

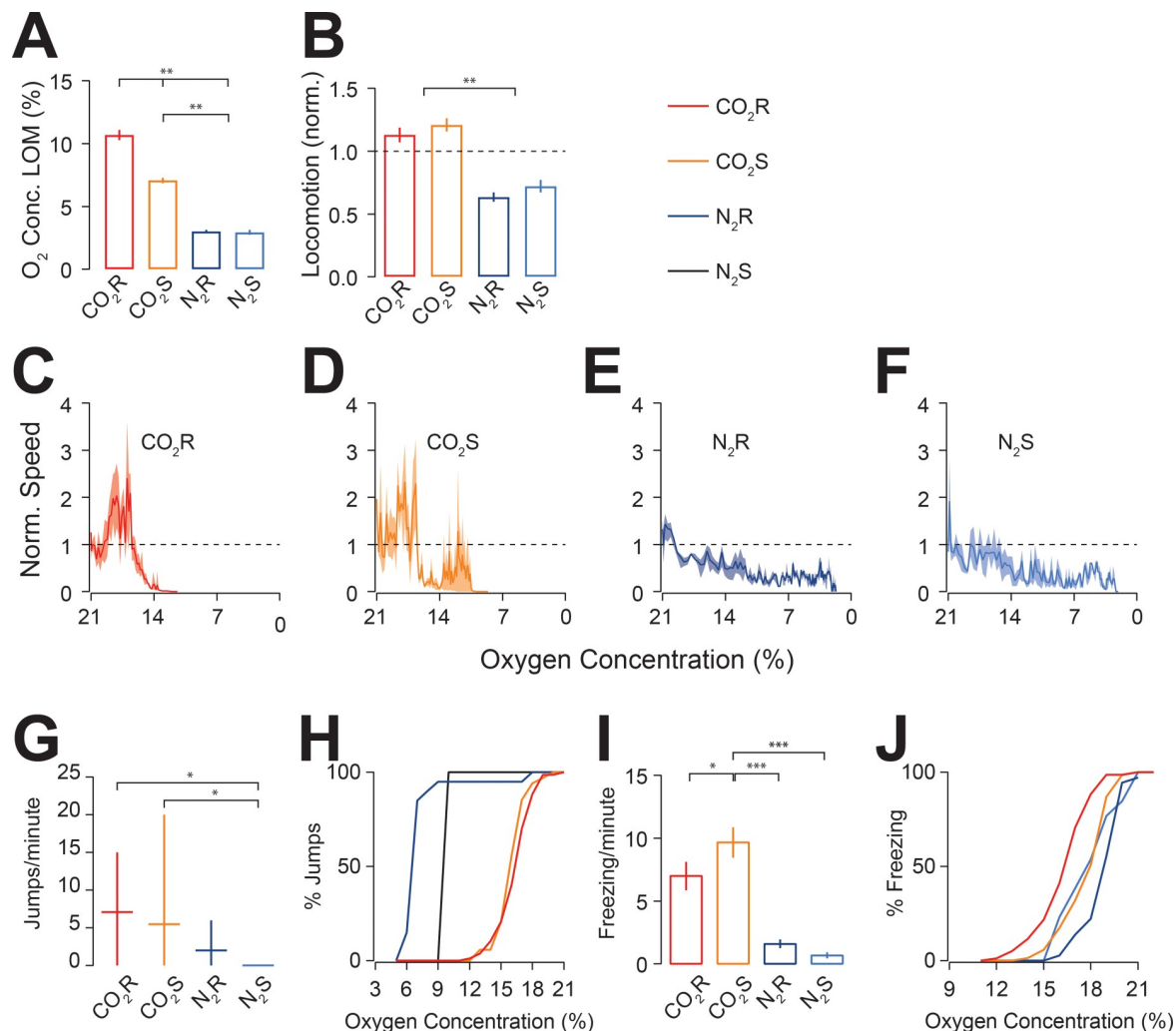
### Results

No animals were excluded from experimentation or analysis. To determine whether nitrogen is less aversive than carbon dioxide as a euthanasia agent for mice, we first assessed the behavioural parameters of groups of non-instrumented C57Bl/6 mice being euthanised in four different treatment groups (CO<sub>2</sub>R; n = 10 cages; female: n = 5; male: n = 5; N<sub>2</sub>R; n = 10 cages; female: n = 5; male: n = 5; CO<sub>2</sub>S; n = 10 cages; female: n = 5; male: n = 5; and N<sub>2</sub>S; n = 10 cages; female: n = 5; male: n = 5). There were no significant differences in the number of animals per cage between the four gas exposure groups (CO<sub>2</sub>R; n = 6 (4–7); N<sub>2</sub>R; n = 6 (5–8); CO<sub>2</sub>S; n = 6 (5–8); N<sub>2</sub>S; n = 5.5 (4–8);  $P = 0.41$ ;  $F = 0.99$ ; one-way ANOVA).

### Locomotion activity

First, we measured locomotor activity of groups of non-instrumented mice during exposure to CO<sub>2</sub> and N<sub>2</sub> with either rapid or slow fill in a euthanasia paradigm. We first investigated the differences in oxygen concentration at loss of motion (LOM), defined as the point when the last animal in the group stopped purposeful movements excluding breathing, which we interpreted as the first stage towards unconsciousness. Interestingly, we found that CO<sub>2</sub>R resulted in LOM at higher oxygen concentrations than CO<sub>2</sub>S (CO<sub>2</sub>R:  $10.7 \pm 0.4\%$ ; CO<sub>2</sub>S:  $7.1 \pm 0.2\%$ ;  $P < 0.01$ ; one-way T-test; Fig 2A). In comparison N<sub>2</sub> groups reached LOM at significantly lower oxygen concentrations than CO<sub>2</sub> ( $P = 0.0058$ ;  $F = 5.0$ ; one-sided ANOVA) however there was no difference between N<sub>2</sub>R and N<sub>2</sub>S groups (N<sub>2</sub>R:  $3.0 \pm 0.1\%$ ; N<sub>2</sub>S:  $2.9 \pm 0.2\%$ ;  $P = 0.073$ ; one-way T-test; Fig 2A).

We then compared the speed of locomotion during gas exposure, normalised to the last 30 seconds of baseline, for each treatment group and plotted it against O<sub>2</sub> concentration. These measures were therefore independent of time, allowing direct comparison of rapid and slow fill groups. We found no difference in speed between treatment groups during the last 30 s of baseline ( $P = 0.43$ ;  $F = 0.96$ ; one-way ANOVA). We found that there were no differences between groups for the average locomotion speed during baseline (CO<sub>2</sub>R:  $0.84 \pm 0.17$  cm/s; N<sub>2</sub>R:  $0.79 \pm 0.20$  cm/s; CO<sub>2</sub>S:  $0.83 \pm 0.23$  cm/s; N<sub>2</sub>S:  $0.81 \pm 0.11$  cm/s;  $P = 0.97$ ;  $F = 0.015$ ; one-way ANOVA). We found that there was a significant increase in locomotion speed for both CO<sub>2</sub>R (ratio of baseline locomotion and gas exposure locomotion:  $1.13 \pm 0.06$ ;  $P = 0.028$ ,



**Fig 2. Effects of CO<sub>2</sub> and N<sub>2</sub> on locomotion, jumping and freezing in grouped mice.** (A) Average (mean  $\pm$  s.e.m) oxygen concentration at loss of motion (LOM). CO<sub>2</sub>R resulted in LOM at higher oxygen concentrations than other groups. CO<sub>2</sub>S resulted in LOM at oxygen concentrations higher than nitrogen groups (\*\*P < 0.01). (B) Average speed (mean  $\pm$  s.e.m), normalised to the last 30 seconds of baseline. Horizontal line indicates the normalisation value for baseline measurements. CO<sub>2</sub> resulted in increased locomotion compared to nitrogen (\*\*P < 0.01). (C-F) Normalised speed in relation to oxygen concentration from the start of gas exposure until LOM for CO<sub>2</sub>R (C), CO<sub>2</sub>S (D), N<sub>2</sub>R (E), and N<sub>2</sub>S (F). (G) Aversive jumps shown as median ( $\pm$  range) of total jumps and inter-quartile range per cage. Both CO<sub>2</sub>R and CO<sub>2</sub>S resulted in significantly more jumps compared to N<sub>2</sub>S (\*P < 0.05). (H) Cumulative curves indicating the relative number of jumps in relation with oxygen concentration of the four treatment groups. (I) Freezing shown as mean ( $\pm$  s.e.m) number of freezing episodes per minute per animal. Mice in CO<sub>2</sub>S froze significantly more times than in the other three groups (\*P < 0.05; \*\*\*P < 0.001). (J) Cumulative curves indicating the relative freezing episodes in relation with the oxygen concentration of the four treatments groups.

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$t = 2.25$ ; two-sided T-test) and CO<sub>2</sub>S ( $1.21 \pm 0.05$ ,  $P = 0.002$ ;  $t = 3.97$ ; two-sided T-test) groups compared to baseline during the first 30s of exposure. Interestingly, we found a significant decrease of activity for both N<sub>2</sub>R ( $0.64 \pm 0.04$ ;  $P < 0.0001$ ;  $t = 9.77$ ; two-sided T-test) and N<sub>2</sub>S ( $0.72 \pm 0.05$ ;  $P < 0.0001$ ;  $t = 5.69$ ; two-sided T-test) groups (Fig 2B). We found no significant difference in locomotion speed between rapid and slow groups for either CO<sub>2</sub> ( $P > 0.05$ ; two-sided T-test) or N<sub>2</sub> ( $P > 0.05$ ; two-sided T-test). We found no difference in locomotion speed between males and females within the different groups ( $P > 0.05$ ; one-way ANOVA). To determine the effect of gas exposure on locomotion, we compared the speed of locomotion between groups from the start of exposure until LOM and normalised to oxygen concentration



(Fig 2C–2F). We found that in both CO<sub>2</sub> groups, there was a transient spike in speed followed by a rapid decline, which followed the same distribution, irrespective of oxygen concentration ( $P = 0.11$ ; Kolmogorov-Smirnov test). Interestingly, for CO<sub>2</sub>S at lower oxygen concentrations, there was a sudden increase in activity not seen with CO<sub>2</sub>R ( $P < 0.05$ ; Kolmogorov-Smirnov test). In contrast, speed declined slowly for both N<sub>2</sub> treatments with no significant differences between groups ( $P > 0.05$ ; Kolmogorov-Smirnov test).

## Jumps

Vertical jumping in mice is considered to be a behavioural response to an aversive environment, independent of pain [44] and has been reported previously in response to CO<sub>2</sub> exposure, interpreted as aversive behaviour [35]. Therefore, we measured the rate of vertical jumps during gas exposure in non-instrumented mice and compared them to baseline conditions as a measure of aversion. We did not observe any other forms of jump, such as forward leaping. We found that jumps were commonly shown and occurred significantly more often when compared to baseline for CO<sub>2</sub>R ( $0.036 \pm 0.08$  jumps min<sup>-1</sup>,  $P = 0.0063$ ; two-sided T-test) and CO<sub>2</sub>S ( $0.03 \pm 0.01$  jumps min<sup>-1</sup>;  $P = 0.0037$ ; two-sided T-test) groups, but not N<sub>2</sub>R ( $P = 0.11$ ; two-sided T-test) or N<sub>2</sub>S ( $P = 0.34$ ; two-sided T-test). Indeed, both CO<sub>2</sub>R and CO<sub>2</sub>S resulted in significantly more jumps than N<sub>2</sub>S (0 jumps;  $P = 0.0056$ ;  $P = 0.0353$ ; respectively one-way ANOVA). However, there was no significant difference between the CO<sub>2</sub>S and N<sub>2</sub>R (0 jumps;  $P > 0.05$ , one-way ANOVA), mice exposed to CO<sub>2</sub>R showed significantly more jumps per minute than in N<sub>2</sub>R group ( $P = 0.0305$ , one-way ANOVA; Fig 2G). No jumps were observed during baseline conditions. To determine the effect of gas exposure on vertical jumps, we compared the occurrence of jumps between groups from the start of exposure until LOM with respect to oxygen concentration (Fig 2H). We found no difference in distribution between treatment CO<sub>2</sub> groups ( $P = 0.35$ ; Kolmogorov-Smirnov test) or between N<sub>2</sub> groups ( $P = 0.10$ ; Kolmogorov-Smirnov test). However, we found that jumps occurred at lower oxygen concentrations for N<sub>2</sub> groups compared to CO<sub>2</sub> ( $P < 0.01$ ; Kolmogorov-Smirnov test).

## Freezing

CO<sub>2</sub> has previously been shown to cause fear responses [6], which can be assessed by freezing behaviour, an immobile state without any movements except respiration whilst the animal is standing with the head raised [6, 9]. Therefore, we investigated if fear responses were a component of the increased aversion by measuring freezing behaviour during gas exposure. We found that mice did not freeze during baseline. Mice in CO<sub>2</sub>S ( $9.7 \pm 1.2$  freezing episodes/minute) froze significantly more often than CO<sub>2</sub>R ( $7.0 \pm 1.1$  freezing episodes/minute;  $P = 0.012$ ; two-sided T-test), N<sub>2</sub>R ( $1.6 \pm 1.4$  freezing episodes/minute;  $P < 0.001$ ; two-sided T-test) and N<sub>2</sub>S ( $0.7 \pm 0.2$  freezing episodes/minute  $P < 0.001$ ; two-sided T-test; Fig 2I). We found no differences between N<sub>2</sub> groups ( $P > 0.05$ ; two-sided T-test). No difference in freezing time was observed (CO<sub>2</sub>R:  $2.6 \pm 0.2$  s; CO<sub>2</sub>S:  $2.8 \pm 0.1$  s; N<sub>2</sub>R:  $2.5 \pm 0.2$  s; N<sub>2</sub>S:  $2.7 \pm 0.3$  s;  $P = 0.44$ ; One-way ANOVA; Data not shown). We did not observe ataxia during the period when animals exhibited freezing behaviour. Interestingly, there was no difference between groups in oxygen concentration for the occurrence of freezing episodes ( $P > 0.05$ ; Kolmogorov-Smirnov test; Fig 2J).

## Electrocortical activity

Unlike CO<sub>2</sub>, N<sub>2</sub> does not have true narcotic effects at normobaric conditions, instead it produces unconsciousness and death by hypoxia. Given that hypoxia could also cause muscle weakness and ataxia we sought to determine whether the reduced locomotion and behaviour

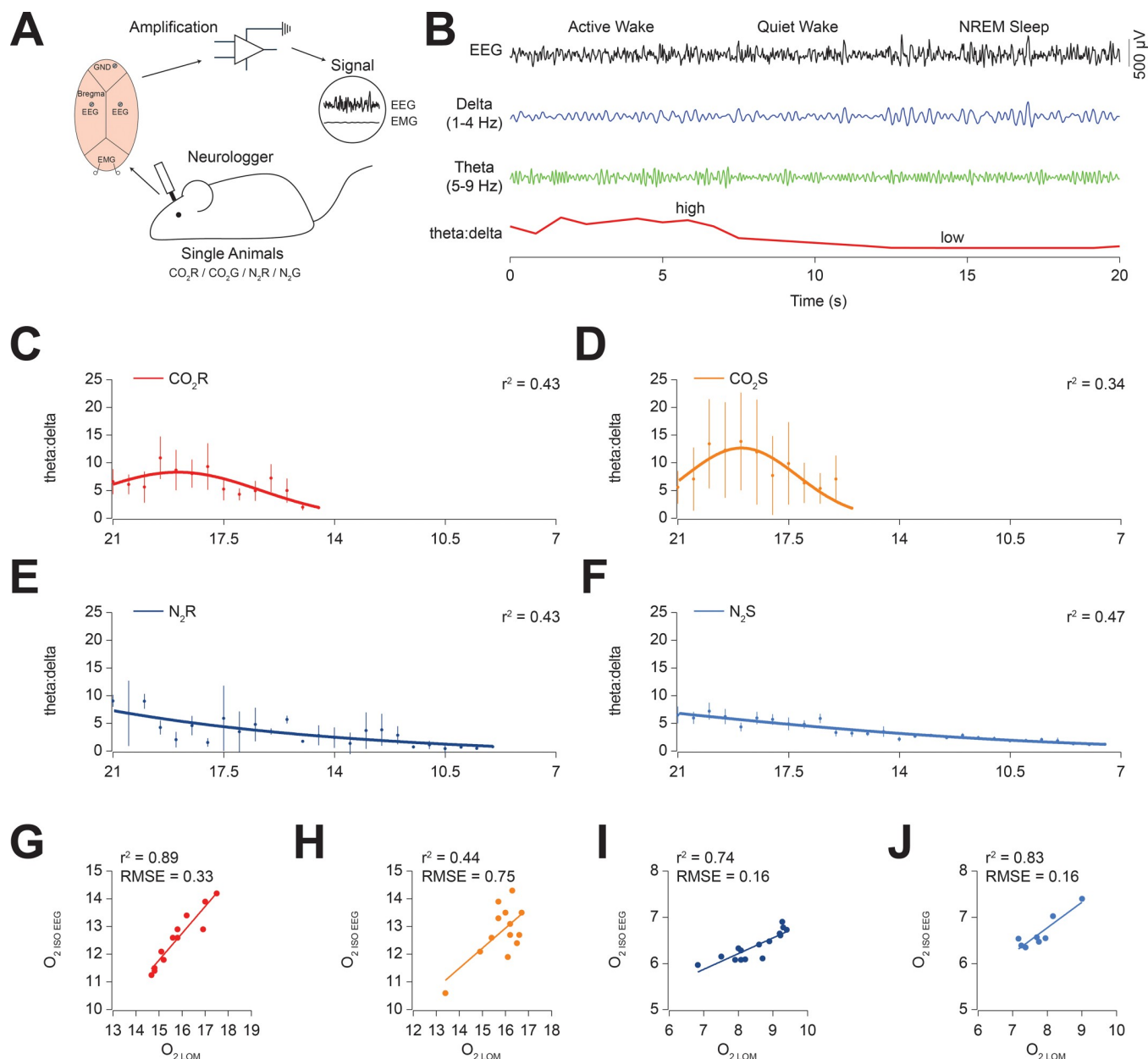
was due to central sedative effects of the hypoxia or peripheral muscle weakness. Therefore, we recorded EEG/EMG in individual chronically instrumented mice during the euthanasia paradigm (Fig 3A; CO<sub>2</sub>R: n = 8 animals; CO<sub>2</sub>S: n = 10 animals; N<sub>2</sub>R: n = 9 animals; N<sub>2</sub>S: n = 10 animals).

In single instrumented animals, we found behavioural patterns (locomotion, jumping and freezing) which paralleled our measurements from grouped animals, suggesting that behaviours were not affected by the animal carrying the Neurologger (S1 Fig). We measured the ratio of theta and delta (TD) oscillations in the EEG, as a measure of neocortical excitation during gas exposure (Fig 3B; ref: [45]). We found that in CO<sub>2</sub> groups there was a significant increase in TD compared to baseline ( $P = 0.015$ ;  $F = 4.1$ ; one-way ANOVA; Fig 3C and 3D) suggesting heightened central neuronal arousal. Baseline values of TD were not significantly different between groups (CO<sub>2</sub>R:  $6.6 \pm 2.3$ ; CO<sub>2</sub>S:  $5.6 \pm 0.9$ ; N<sub>2</sub>R:  $8.1 \pm 1.1$ ; N<sub>2</sub>S:  $6.5 \pm 1.5$ ;  $P = 0.61$ ;  $F = 0.61$ ; one-way ANOVA). Interestingly, TD increased significantly more for CO<sub>2</sub>S compared to CO<sub>2</sub>R (measured at the curve apex; CO<sub>2</sub>R:  $8.3 \pm 1.6$ ; CO<sub>2</sub>S:  $12.7 \pm 1.3$ ;  $P = 0.048$ ;  $t = 2.15$ ; d.f. = 15; two-sided T-Test). By contrast, TD decreased uniformly from baseline, inversely proportional to O<sub>2</sub> concentration in N<sub>2</sub> groups ( $P = 0.015$ ;  $F = 4.1$ ; one-way ANOVA; Fig 3E and 3F). This data suggests that mice in N<sub>2</sub> groups experienced central neuronal depression as a result of decreasing O<sub>2</sub> concentration. Given the different O<sub>2</sub> concentrations of LOM between CO<sub>2</sub> and N<sub>2</sub> groups, we used the EEG to determine O<sub>2</sub> concentrations at the time of brain death determined by isoelectric EEG (ISO-EEG). We then compared these values to O<sub>2</sub> concentrations at LOM as a measure of the reliability of the euthanasia method. As expected, we found that brain death occurred at higher O<sub>2</sub> concentrations for CO<sub>2</sub> groups compared to N<sub>2</sub> groups (CO<sub>2</sub>R:  $12.6 \pm 0.3$ ; CO<sub>2</sub>S:  $12.8 \pm 2.7$ ; N<sub>2</sub>R:  $6.4 \pm 0.1$ ; N<sub>2</sub>S:  $6.7 \pm 0.1$ ;  $P < 0.0001$ ;  $F = 286.3$ ; one-way ANOVA). Interestingly, we found a high correlation between LOM and ISO-EEG for CO<sub>2</sub>R ( $r^2 = 0.89$ ), N<sub>2</sub>R ( $r^2 = 0.74$ ) and N<sub>2</sub>S ( $r^2 = 0.83$ ) groups but low correlation for CO<sub>2</sub>S ( $r^2 = 0.44$ ). Furthermore, the fits were significantly different between CO<sub>2</sub> groups, as measured from the root mean square errors (RMSE; CO<sub>2</sub>R: 0.33; CO<sub>2</sub>S: 0.75;  $P = 0.03$ ;  $t = 2.3$ ; d.f. = 23; two-way T-test) suggesting less predictability for the point of death when using CO<sub>2</sub>S.

## Discussion

The AVMA guidelines state that the use of humane euthanasia techniques should induce the most rapid, painless and distress-free death as possible [23]. Identifying pain during mice euthanasia is a critical end-point and currently, no objective measures have been validated for assessing pain associated with euthanasia [30]. However previous studies have identified markers of aversion [35] and fear [6] in response to CO<sub>2</sub> exposure. Since inert gases are colourless, odourless and non-irritant, we hypothesised that N<sub>2</sub> would be less aversive than CO<sub>2</sub> in a gas euthanasia paradigm.

We found that mice exposed to CO<sub>2</sub> showed behavioural excitation, manifested by increased locomotion, freezing and jumping compared to baseline, which others have interpreted as signs of aversion [11, 25, 35, 46]. Thomas et al., [35] previously reported jumping in response to CO<sub>2</sub> exposure which the authors interpreted as escape behaviour. Based on an observation of exaggerated jumping with the addition of N<sub>2</sub>, they hypothesised that jumping resulted from hypoxia. However, in our study, we noted more numerous jumps with CO<sub>2</sub> compared to N<sub>2</sub>, and at higher O<sub>2</sub> concentrations, suggesting that the jumps observed with CO<sub>2</sub> are primarily a result of CO<sub>2</sub> exposure, although hypoxia may also cause jumping [35]. This aversion may be due to carbonic acid formation in the nasal mucous membranes causing irritation [6, 47]. Additional brainstem circuits have been identified which produce arousal



**Fig 3. CO<sub>2</sub> causes central neurological excitation whereas N<sub>2</sub> causes central neurological depression.** (A) Schematic of recording. From bottom going clockwise: Single euthanased mice were used and underwent euthanasia with CO<sub>2</sub> or N<sub>2</sub> at either rapid fill (R—80% volume fill minute<sup>-1</sup>) or slow fill (G—30% volume fill minute<sup>-1</sup>). EEG and EMG electrodes were chronically implanted and animals allowed to recover for seven days. Data were acquired on a portable wireless Neurologger at 200 Hz and data downloaded after the experiment onto a computer. (B) Representative sample of a 20 s EEG recording from a mouse recorded during baseline. Filtered delta (1–4 Hz; blue) and theta (5–9 Hz; green) signals are shown below. Note the difference in amplitude between the delta and theta signals changing over time. The theta:delta power ratio (TD) shown in red on the bottom line. Note the high TD when theta amplitude is high and low TD when delta amplitude is high. (C, D, E, F) TD values with respect to O<sub>2</sub> concentration for CO<sub>2</sub>R (C), CO<sub>2</sub>S (D), N<sub>2</sub>R (E) and N<sub>2</sub>S (F). Note the initial increase in TD for CO<sub>2</sub> groups compared to the slow reduction in N<sub>2</sub> groups. (G, H, I, J) Linear regressions of O<sub>2</sub> concentration at isoelectric EEG (ISO-EEG) against O<sub>2</sub> concentration at LOM for CO<sub>2</sub>R (G), CO<sub>2</sub>S (H), N<sub>2</sub>R (I) and N<sub>2</sub>S (J). Note the increased scatter of points for CO<sub>2</sub>S.

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from natural sleep in response to CO<sub>2</sub> [48]. These identified neurones in the parabrachial nucleus are strong modulators of neocortical excitation [49]. Thus, there are multiple potential mechanisms for producing the neurological and behavioural excitation that we observed. However, both CO<sub>2</sub> and hypoxia are likely to be factors in inducing this behaviour.

Interestingly, they also noted a decreased jumping with the addition of  $N_2O$  to  $CO_2$  due to an increased systemic absorption of  $CO_2$  [35]. We did not observe reduced jumping with rapid fill carbon dioxide but we cannot quantitatively compare absorption rates in the two studies. However, we did observe that  $CO_2S$  resulted in increased freezing compared to  $CO_2R$ , suggesting a fear response to its exposure [6, 9]. Furthermore, we showed heightened excitation of brain activity to slow fill  $CO_2$  compared to rapid fill, suggesting that the duration of exposure to  $CO_2$  might be a more important factor than the concentration of  $CO_2$  itself for behavioural and neurological excitation. Interestingly, we observed no jumping or freezing behaviour during baseline activity. However, other strains of mice that show increased stress responses compared to C57Bl6 mice [50] might exhibit this behaviour during the baseline.

The AVMA guidelines report that “an optimal flow rate for  $CO_2$  euthanasia systems should displace 10% to 30% of the chamber or cage volume/min”, however the use of  $CO_2$  at high flow is not specifically contraindicated in the AVMA recommendations. Rather, it is considered unacceptable to place a conscious animal inside a 100%  $CO_2$  pre-filled chamber [23]. Previous investigation into different fill rates of  $CO_2$  concluded that slow fill did not lessen behavioural, cardiovascular or histological markers of distress in mice [30]. Taken together, these data suggest that use of rapid fill  $CO_2$  may result in less aversion than slow fill. Indeed, rapid fill might be beneficial compared to slow fill, which may cause a longer time until loss of consciousness and death, and prolonged period of dyspnoea, anxiety or distress [12, 30].

Conversely, mice exposed to  $N_2$  decreased their locomotor activity compared to baseline, suggesting a sedative effect of reduced atmospheric oxygen, as suggested by previous work [51]. Reduced locomotion speed did not result from prolonged freezing, since freezing episode duration was the same between groups. We further found that behavioural excitation or retardation was mirrored by neurophysiological activity. Based on behavioural assessment alone, one might surmise that reduced locomotion and jumping during  $N_2$  exposure might result from muscle weakness induced by hypoxia. However, we showed that brain activity depression occurs at the same time, raising the likelihood that the reduced behaviour is mediated by brain activity. However, a direct causality remains to be determined. Hypoxia in mice causes air hunger [35] as well as in other species [19]. Although we did not measure respiratory values in our study, other behavioural and electrophysiological variables were low at hypoxic conditions for animals exposed to  $N_2$ . The impact of air hunger on animal welfare in this context requires further evaluation. Furthermore, freezing was not dependent on oxygen concentration between groups and therefore is unlikely to be due to hypoxia in the  $N_2$  groups, although previous reports of argon induced hypoxia showed that it was aversive to mice [10], which would be consistent with our results.

Exposure to  $CO_2$  is reported to result in pain in humans [3, 16] which is assumed to occur in rodents as well. Pain was not an endpoint for our study and is considered difficult to gauge in mice during acute conditions [20]. Based on these studies documenting pain and irritation following  $CO_2$  exposure in humans, it is likely that mice in this study also experienced pain, although this cannot be directly assessed. The degree of distress from this pain results from the pain intensity and its duration. In that regards, slow fill regimes in which animals are exposed to  $CO_2$  for longer are likely to result in more distress, although this requires further investigation. Hypoxia also causes pain manifested as headache in humans [52], although this is typically associated with less severe hypoxia than used in this study. Furthermore, the onset of pain takes longer, in the region of several hours. Whether pain manifests during very short-term hypoxia as experienced in the  $N_2$  groups remains to be determined.

Our results show that  $CO_2$  slow fill does not prevent aversive behaviour such as jumping or increased locomotion and moreover it caused more freezing episodes than rapid fill  $CO_2$ . The decreased incidence of freezing during rapid fill  $CO_2$  indicates a low concentration threshold

for the fear component of CO<sub>2</sub> exposure, as previously reported [6]. Therefore, rapid fill would reduce the time from this threshold until a hypnotic/narcotic effect, therefore potentially reducing the number of jumps and freezing episodes observed. This would be supported further by our observation that freezing was not dependent on fill rate. A significant proportion of freezing from CO<sub>2</sub> exposure is a chemical effect, as indicated by the lower incidence in nitrogen groups. Furthermore, mice showed increased locomotor activity at lower oxygen concentrations in the CO<sub>2</sub> slow fill group, suggesting that this increases the likelihood of animals becoming hypoxic before losing consciousness.

## Conclusion

Our study demonstrates that N<sub>2</sub> produces reduced behavioural and electrophysiological excitation during euthanasia compared to CO<sub>2</sub>. However, N<sub>2</sub> may still cause some degree of fear response. Furthermore, rapid fill CO<sub>2</sub> might be less aversive than slow fill. Further work is required to assess other physiological and behavioural parameters, such as nociception, to determine if N<sub>2</sub> is a suitable euthanasia agent for mice.

## Supporting information

**S1 Fig. Effects of CO<sub>2</sub> and N<sub>2</sub> on locomotion, jumping and freezing in single instrumented mice.** Behavioural markers in the four treatment groups: CO<sub>2</sub> rapid fill (CO<sub>2</sub>R), CO<sub>2</sub> slow fill (CO<sub>2</sub>S), nitrogen rapid fill (N<sub>2</sub>R) and nitrogen slow fill (N<sub>2</sub>S). (A) Average (mean  $\pm$  s.e.m) oxygen concentration at loss of motion (LOM). CO<sub>2</sub>R resulted in LOM at higher oxygen concentrations than other groups. CO<sub>2</sub>S resulted in LOM at oxygen concentrations higher than nitrogen groups (\*\*P < 0.01; \*\*\*\*P < 0.0001). (B) Average speed (mean  $\pm$  s.e.m), normalised to the last 30 seconds of baseline. Horizontal line indicates the normalisation value for baseline measurements. CO<sub>2</sub> resulted in increased locomotion compared to nitrogen (\*\*P < 0.01). (C-F) Normalised speed with respect to oxygen concentration from the start of gas exposure until LOM for CO<sub>2</sub>R (C), CO<sub>2</sub>S (D), N<sub>2</sub>R (E), and N<sub>2</sub>S (F). (G) Vertical jumps shown as median of jumps per animal and inter-quartile range per cage. Both CO<sub>2</sub>R and CO<sub>2</sub>S resulted in significantly more jumps compared to N<sub>2</sub>S (\*\*\*P < 0.001; \*\*\*\*P < 0.0001) (H) Cumulative curves indicating the relative number of jumps in relation with oxygen concentration of the four treatment groups. (I) Freezing shown as mean ( $\pm$  s.e.m) number of freezing episodes per minute per animal. Mice in CO<sub>2</sub>S froze significantly more times than in the other three groups (\*\*P < 0.01). (J) Cumulative curves indicating the relative freezing episodes in relation with the oxygen concentration of the four treatment groups. (TIF)

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